International Journal of Food Microbiology 157 (2012) 360–367

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

The infection capacity of *P. expansum* and *P. digitatum* on apples and histochemical analysis of host response

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ARTICLE INFO

Article history:
Received 7 March 2012
Received in revised form 4 June 2012
Accepted 7 June 2012
Available online 13 June 2012

Keywords:
Blue mould
Green mould
Maturity stage
Defence
Lignin
Wound healing

ABSTRACT

Fruit ripening is a complex process that involves a variety of biochemical changes and is also associated with increased susceptibility to pathogens. The present study determined the effects of fruit maturity and storage conditions on the infection capacity of a host (*P. expansum*) and non-host (*P. digitatum*) pathogen on apple. A range of inoculum concentrations and two different storage temperatures were utilized. Exposure to *P. expansum* at 20 °C resulted in significant differences in rot dynamics in apples collected at the earliest harvest date compared to all later harvest dates and inoculum concentrations assayed. Greater differences in infection capacity between harvests were obtained when fruit was stored at low temperature (0 °C). In contrast, *P. digitatum* was able to infect apples only under specific conditions and disease symptoms were limited to the initial wound inoculation site. When apples were resistant to *P. digitatum*, a visible browning reaction around the infection site was observed. Histochemical analyses of tissues surrounding the wound site were conducted. A positive reaction for lignin was observed in immature apples as early as 1 day after inoculation with either pathogen. Experiments conducted with the non-host pathogen indicated that lignification was an essential component of resistance in apples harvested prior to maturity or at commercial maturity. Apples harvested at an over-mature stage and inoculated with *P. digitatum* did not show evidence of staining for lignin until 7 days post-inoculation. Control samples only showed positive reaction in immature harvest. Results demonstrated that the maturity stage of fruit is an important factor in apple resistance to both *P. expansum* and *P. digitatum* and that lignin accumulation seems to play an important role when resistance is observed. Moreover, this is the first report demonstrating that *P. digitatum*, a non-host pathogen, has a limited capacity to infect apples.

1. Introduction

Blue mould, caused by *Penicillium expansum*, and green mould, caused by *Penicillium digitatum*, are the most important postharvest diseases of apples and citrus fruits, respectively. Both pathogens are necrotrophs that require wounds to enter the fruit (Kavanagh and Wood, 1967; Spotts et al., 1998). Mechanical injury caused during harvesting and postharvest handling provides an optimal locus for infection. The use of chemical fungicides is one of the primary means of controlling these postharvest diseases; however, fungicides may have a negative impact on the environment and both human and animal health. Their long-term use also leads to the development of fungicide-resistant strains. These problems have motivated the search for alternative approaches and the study of host–pathogen interactions to provide a better understanding of the virulence mechanisms of the pathogens as well as the defence responses of the hosts in order to design new and safer control strategies.

A host–pathogen interaction may be categorized as compatible if a pathogen overcomes plant defence barriers and establishes disease symptoms, whereas in a non-host or incompatible pathogen interaction, plants deploy an array of defences that prevent or significantly limit pathogen growth (Glazebrook, 2005). Resistance responses involve a complex and dynamic communication system that is established during the first steps of infection.

One of the most rapid defence reactions is the oxidative burst that is characterized by a rapid and transient accumulation of reactive oxygen species (ROS) (Torres et al., 2006) composed primarily of superoxide anion and hydrogen peroxide at the site of the invasion (Apel and Hirt, 2004). Research has shown that avirulent pathogens induce a biphasic ROS production in plants, consisting of a low amplitude first phase, followed by a much higher and sustained accumulation during the second phase (Lamb and Dixon, 1997; Torres et al., 2006). However, only the first phase has been detected during interactions with virulent pathogens (Bolwell et al., 2001). In oranges inoculated with *P. digitatum* the production of ROS seems to be

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doi:10.1016/j.ijfoodmicro.2012.06.005

suppressed whereas inoculation with *P. expansum*, a closely related non-host species, triggers the production of ROS at attempted penetration sites (Macarisin et al., 2007).

ROS production also has been associated with the formation of physical defensive barriers against the pathogens (Huckelhoven and Kogel, 2003) established at the site of the infection. Changes in gene expression involving increased expression of phenylpropanoid metabolism genes have also been detected in tissues undergoing a resistance response (Hutcheson, 1998). Phenylalanine ammonia lyase (PAL) is a key enzyme in this pathway, and is directly involved in the synthesis of phenols and lignin (Yao and Tian, 2005). PAL contributes to the disease resistance response in many fleshy fruits (Singh et al., 2010). Vilanova et al. (2012) demonstrated a positive reaction for lignin in immature oranges in both host (*P. digitatum*) and non-host (*P. expansum*) pathogen interactions.

Plant defence strategies against pathogen invasion may be modulated by fruit ripening (Su et al., 2011) which is itself a complex, developmentally regulated process encompassing alterations in gene expression and chemical and physiological changes (Cantu et al., 2008). However, some questions remain unanswered as to how fruit maturity may affect the infection capacity of both host and non-host pathogens. Torres et al. (2003) reported that apples harvested 7 days after commercial harvest were more susceptible to *P. expansum* than apples harvested 7 days before commercial harvest. Beno-Moualem and Prusky (2000) correlated higher levels of ROS found in unripe avocado with a lower susceptibility to *Colletotrichum gloeosporioides* compared to ripe fruit. In contrast, Davey et al. (2007) reported that the susceptibility of different apple genotypes to *Botrytis cinerea* decreased when the harvest date was extended.

The aim of the present study was to investigate the infection capacity of the host, *P. expansum* (compatible), and the non-host, *P. digitatum* (incompatible) pathogens in 'Golden Smoothee' apples at different (i) maturity stages; (ii) inoculum concentrations, and (iii) storage temperatures.

The infection capacity studies were combined with a histochemical analysis of apple fruit tissues at the site of inoculation to characterize the accumulation of suberin and lignin in order to define their role in host resistance against both pathogens.

2. Materials and methods

2.1. Fruits

'Golden Smoothee' apples were harvested at different maturity stages from August to October, 2009 (six harvests ranging from immature to over-mature) from a commercial orchard in Mollerussa (Catalonia, Spain). Harvests 1 and 2 were considered as prior to commercial maturity (immature fruit), harvests 3–5 were considered as commercial maturity (mature fruit), and harvest 6 was considered as past maturity (over-mature fruit). Apples were used immediately after harvest. Data obtained for quality as described below confirmed that the harvest dates represented different levels of maturity.

2.2. Fungal cultures

P. expansum CMP-1 and *P. digitatum* PDM-1 are the most aggressive isolates from our collection capable of infecting pome fruits and citrus, respectively. They are maintained on potato dextrose agar medium (PDA; 200 mL boiled potato extracts, 20 g dextrose, 20 g agar and 800 mL water) and periodically grown on wounded pome fruits (*P. expansum*) or citrus (*P. digitatum*) and then reisolated to maintain virulence. Conidia from 7- to 10-day-old cultures grown on PDA were collected by rubbing the surface of the agar with sterile glass rod. The concentration was determined with a haemocytometer and diluted to different concentrations (10^7, 10^6, 10^5 or 10^4 conidia/mL) and then used for the determination of infection capacity.

2.3. Infection capacity

The effects of fruit maturity, inoculum concentration, and storage temperatures were assessed for both the compatible interaction (*P. expansum*-apples) and the incompatible interaction (*P. digitatum*-apples). Apples were washed thoroughly with tap water and allowed to dry before artificial inoculation. Apples were wounded with a nail (1 mm wide and 2 mm deep) and inoculated with 15 μL of an aqueous conidial suspension of either pathogen at four different concentrations; 10^7 and 10^6 conidia/mL are considered in this work as high inoculum concentrations, and 10^5 and 10^4 conidia/mL are considered as low inoculum concentrations. The infection capacity of each pathogen was assessed at two different storage temperatures (0 and 20 °C) and 85% relative humidity. The diameter of rot was measured over the duration of each experiment in order to obtain information on the rot dynamics of each pathogen as affected by inoculum concentration, temperature, and fruit maturity. Five apples constituted a single replicate and each treatment was repeated four times.

2.4. Determination of quality parameters

Colour development, flesh firmness, starch index, soluble solids and acidity were determined to evaluate the effects of different harvest dates on fruit quality.

Colour was measured using hue values, which were calculated from a^* (red-greenness) and b^* (yellow-blueness) parameters measured with a CR-200 chromameter (Minolta, Japan) on both the exposed and the shaded sides of each fruit, using standard CIE illuminant and 8 mm viewing aperture diameter. Flesh firmness was measured on two opposite sides of each fruit with a penetrometer (Effegi, Milan, Italy) equipped with an 11 mm diameter plunger tip. Total soluble solids content (TSS) and titratable acidity (TA) were assessed in juice using a refractometer (Atago, Tokyo, Japan) and by titration of 10 mL of juice with 0.1 N NaOH and 1% phenolphthalein as an indicator. Starch hydrolysis was rated visually using a 1–10 EUROFRU scale (1, full starch; 10, no starch) (Planton, 1995), after dipping of cross-sectional fruit halves in 0.6% (w/v) I_2–1.5% (w/v) KI solution for 30 s. Data on maturity indexes represent the mean of 20 individual fruits.

2.5. Histochemical tests

The development of resistance was studied by wounding 'Golden Smoothee' apples at three maturity stages: immature (harvest 1), commercial (harvest 4) and over-mature (harvest 6). Apples were inoculated with *P. expansum* or *P. digitatum* at either 10^7 or 10^4 conidia/mL. Control fruits were wounded but not inoculated. Fruits were stored at 20 °C and 85% RH and samples collected for histochemical analyses at 1, 3, 5, 7, and 9 days.

At each collection time, excised peel and pulp tissue cylinders (8 mm inside diameter and 4 mm deep) encompassing the wound site were infiltrated with FAA (formalin, glacial acetic acid, 96% ethanol, and water 10:5:50:35 v/v) and fixed for up to 48 h. Cylinders were dehydrated in an ethanol-xylene series, embedded in paraffin, sectioned transversely along the long axis at a thickness of 20 μm with a rotary microtome and fixed to glass-slides with Haupt adhesive and heat. Sections were deparaffinised with xylene and brought to miscibility with water to apply the following histochemical tests:

I. A Maüle reaction for lignin was performed according to the method described by Thomson et al. (1995) with slight modifications. Sections on slides were stained with 1% (v/v) aqueous potassium permanganate for 15 min, rinsed three times with distilled water (30 s each rinse), placed in 1% (v/v) HCl for 4 min, rinsed in water and then placed in 0.025% (v/v) ammonia for 5 s. The sections were rinsed in distilled water for

1 min, followed by 70% ethanol for 2 min. The sections were mounted in glycerine.
The Maüle test can differentiate apparent syringyl (S) moieties (red) from the p-hydroxyphenyl (H) and guaiacyl (G) components (brown) and allows the qualitative evaluation of lignin monomer composition in cell types (Guillaumie et al., 2010).

II. A toluidine blue O test for lignin was performed according to the method described by Krishnamurthy (1999). Sections on slides were stained in aqueous toluidine blue O solution, pH 4.4 (0.05% stain in benzoate buffer [0.25 g benzoic acid and 0.29 g sodium benzoate in 200 mL water]). They were then washed and mounted in distilled water.
III. A Sudan IV test for suberin was performed according to the method from Johansen (1940) with slight modifications. Sections on slides were immersed in Sudan IV solution for 10 min. The Sudan IV solution was prepared by adding 50 mL of glycerine to 50 mL of a saturated solution of Sudan IV in 95% ethanol and filtering. Sections were rinsed in 70% ethanol and then mounted in glycerine.
IV. An Aniline blue test for callose was performed according to the method described by Krishnamurthy (1999) with slight modifications. Sections on slides were stained in aqueous aniline blue solution 0.005% (w/v) for 10 min. The sections were rinsed and mounted in distilled water.

Samples were analysed with both a Leica MZ16F stereoscope and Leica DM5000 microscope. Images were acquired using a Leica colour digital camera (Leica DFC 420).

Apples infected with *P. expansum* at 10^7 and 10^4 conidia/mL showed complete rot development after 5 days of incubation so histochemical analyses were not conducted after this point. Therefore, 1 day after inoculation was considered a short-period response because at this time, samples could be excised intact from the fruit and histochemically examined. Seven days after inoculation was considered a long-period response for samples inoculated with *P. digitatum*.

2.6. Data analysis

The average diameters (cm) at each time of measurement (day) were plotted and growth rates (cm/day) were obtained from the slopes by linear regression using Microsoft Excel (Microsoft Corporation, USA). This method assumes that once the lag phase has passed, growth starts immediately at its maximum rate (cm/day). The lag phase was cut off by the linear growth zone from the initial inoculum diameter. The linear regression method was preferred over sigmoidal curve fittings since no stationary phase was observed for all growth curves because the maximum diameter possible is the fruit diameter and high correlations were obtained with this simple method in other studies (Baert et al., 2007b; Lahlali et al., 2005; Pardo et al., 2005). The uncertainty of the regression was assessed by calculating the adjusted correlation coefficient R^2.

Data collected on the fruit quality parameters, initial day of visible rot, and lesion growth rate (cm/day) were analysed for statistical significance $P<0.05$ using analysis of variance (ANOVA) with the statistical package SAS (Microsoft). Student–Newman–Keuls (SNK) test for separation of means was performed on all parameters found to be significant in the ANOVA.

3. Results

3.1. Changes in quality parameters

Significant differences in maturity indexes were found between harvest dates (Table 1).

Apple maturity stages showed a significant decreased in flesh firmness as the harvest date advanced. In contrast, total soluble solids became higher with harvest date. Acidity only showed significant differences between the greenest harvest and the others. The most useful quality parameter to define the maturity stage in apples is starch index and in this case was the parameter that showed the most important differences between maturity stages of apples. (a^*+b^*) parameter indicated that skin colour was changing from green to yellow, but in that case was not a good parameter to indicated the apple maturity because only significant differences between over-mature and the other harvests were obtained.

3.2. Effect of maturity stage and inoculum concentration on the infection capacity on the compatible interaction at 20 °C

For all harvest dates and all inoculum concentrations, lesion development of 'Golden Smoothee' apples inoculated with *P. expansum* and incubated at 20 °C always displayed a linear growth pattern (Fig. 1).

Only harvest 1 (least mature) showed a different growth pattern from the other harvests. There were no differences between commercially mature and over-mature harvested fruits. Differences between the immature fruits and the fruit collected at other harvests were more pronounced at lower inoculum concentrations. After 12 days of inoculation, lesion diameter averages for the greenest harvest at 10^7, 10^6, 10^5 and 10^4 conidia/mL were approximately 5.2, 5, 3.5 and 1.2 cm respectively; for the other harvests, lesion diameters were around 6 cm at all inoculum concentrations tested.

Statistical analysis indicated that the lesion growth rate was significantly different between inoculum concentrations only for the greenest harvest (data not shown); however, no differences were found at the other harvests. Differences in the time elapsed prior to the first visible symptoms of decay (Table 2) were most pronounced at the lowest inoculum concentration tested. The first visible rot symptoms appeared earlier at high concentrations (10^7 and 10^6 conidia/mL: 2 days) than at low inoculum concentration (10^4 conidia/mL: 2–4 days). However, for the over-mature harvest, no significant differences were found in this parameter (data not shown).

The statistical analysis of growth rate (Table 2) showed that fruit harvested at the immature stage was significantly different from fruit harvested at commercial maturity or over-mature stages for all inoculum concentrations tested. At the higher inoculum concentrations (10^7 and 10^6 conidia/mL), no significant differences were observed between harvest dates. The most important differences between harvests for the time elapsed until the first visible symptoms or rot were found for an inoculation dose of 10^4 conidia/mL: harvest 1 (4 days) and 6 (2 days).

3.3. Effect of maturity stage and inoculum concentration on the infection capacity on the compatible interaction at 0 °C

P. expansum lesion development exhibited a linear pattern of growth at 10^7, 10^6 and 10^5 conidia/mL for all harvest dates except harvest 1 (Fig. 2A–C). In contrast, the lowest inoculum concentration displayed an exponential growth pattern for all harvest dates (Fig. 2D).

Table 1
Effect of harvest date on flesh firmness, malic acid content, soluble solids, starch index and (a^*+b^*) parameter on "Golden Smoothee" apples. Harvest dates with the same letter are not statistically different ($P<0.05$) according to the SNK test.

Harvest	Date	Flesh firmness (N)	Titratable cidity (g/L malic acid)	Total soluble solids (TSS in %)	Starch index	(a^*+b^*)
1	14/08/09	77.2 a	5.7 a	7.6 d	1.8 e	24.4 b
2	28/08/09	74.0 ab	4.7 b	11.8 c	3.5 d	25.6 b
3	04/09/09	71.0 bc	4.1 b	11.3 d	6.1 c	23.5 b
4	10/09/09	68.3 c	4.5 b	13.0 b	8.1 b	26.0 b
5	19/09/09	66.1 c	4.4 b	13.7 a	8.1 b	26.0 b
6	02/10/09	39.6 d	4.5 b	13.2 b	10.0 a	38.0 a

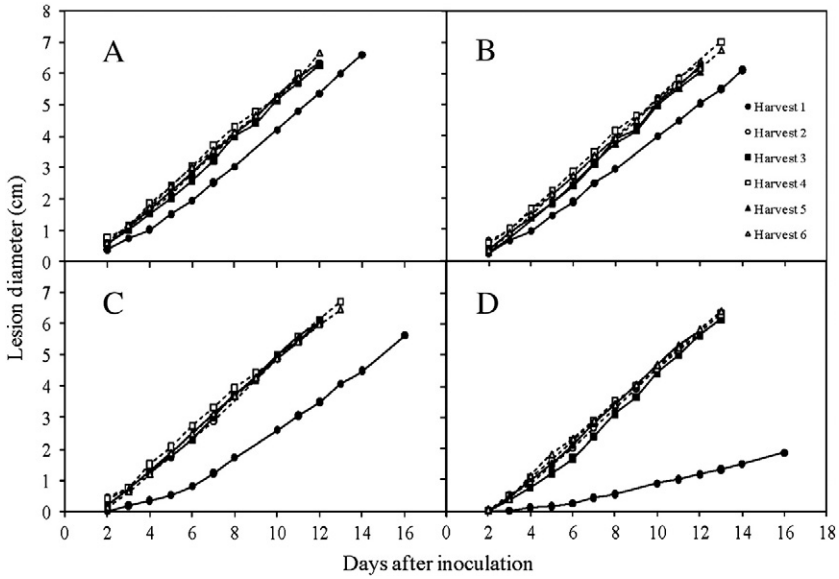

Fig. 1. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 20 °C and 85% HR storage conditions. "Golden Smoothee" apples were harvested at six different dates and inoculated with *Penicillium expansum* at four different inoculum concentrations: 10^7 conidia/mL (A), 10^6 conidia/mL (B), 10^5 conidia/mL (C) and 10^4 conidia/mL (D). Each point represents the mean of 20 fruit.

At higher inoculum concentrations, only harvest 1 (least mature) showed a different growth pattern than the other harvests. However, at lower inoculum concentrations (10^5 and 10^4 conidia/mL) the effect of harvest was more pronounced and three different groups of harvest behaviour could be separated (harvest 1; harvests 2 and 3; harvests 4–6). After 84 days of incubation at 10^7 and 10^6 conidia/mL, lesion diameter averages were approximately 5.5 and 4.5 cm, respectively. Meanwhile, at lower inoculum concentration, rot diameter averages were approximately 4.1 and 3.4 cm, respectively.

Statistical analysis shows no differences in the growth rate between inoculum concentrations at any harvest dates (data not shown); however, significant differences were found for the visible initial rotting day. The first visible symptoms of decay appeared earlier at high inoculum concentrations (10^7 conidia/mL: 21 days) than at low inoculum concentrations (10^4 conidia/mL: 29.7–42 days) (Table 2).

For growth rate, at 10^7 and 10^4 conidia/mL, immature harvests showed significant differences from commercially mature and over-mature harvests; and at 10^6 and 10^5 conidia/mL, only harvest 1 (the least mature harvest) showed significant differences from commercially mature and over-mature harvests, whereas harvest 2 could not be considered different (Table 2). For visible initial rotting day, at 10^7 conidia/mL, no significant differences between harvests were found, and for the other inoculum concentrations, the first three harvests started to rot significantly earlier than the others.

Table 2
Growth rates and visible initial rotting day of *Penicillium expansum* in "Golden Smoothee" apples at four different inoculum concentration, six different harvests and two different storage temperatures. For each inoculum concentration, harvests with different letters are statistically different according to the SNK test ($P<0.05$).

Inoculum concentration	Harvest	20 °C		0 °C	
		Growth rate (cm/day)	Visible initial rotting day (days)	Growth rate (cm/day)	Visible initial rotting day (days)
10^7	1	0.543 b	2.0 a	0.078 c	21.0 a
	2	0.583 a	2.0 a	0.087 b	21.0 a
	3	0.583 a	2.0 a	0.097 a	21.0 a
	4	0.588 a	2.0 a	0.102 a	21.0 a
	5	0.589 a	2.0 a	0.100 a	21.0 a
	6	0.592 a	2.0 a	0.097 a	21.0 a
10^6	1	0.503 b	2.0 a	0.071 c	28.0 a
	2	0.587 a	2.0 a	0.087 b	28.0 a
	3	0.593 a	2.0 a	0.092 ab	28.0 a
	4	0.588 a	2.0 a	0.098 a	22.0 b
	5	0.589 a	2.0 a	0.097 a	21.0 c
	6	0.589 a	2.0 a	0.098 a	21.0 c
10^5	1	0.460 b	3.0 a	0.065 c	35.0 a
	2	0.579 a	2.0 b	0.082 b	35.0 a
	3	0.601 a	2.0 b	0.090 b	35.0 a
	4	0.582 a	2.0 b	0.101 a	29.0 b
	5	0.600 a	2.0 b	0.100 a	28.0 c
	6	0.591 a	2.0 b	0.102 a	28.0 c
10^4	1	0.160 b	4.0 a	0.068 c	42.0 a
	2	0.589 a	3.0 b	0.084 b	42.0 a
	3	0.602 a	3.0 b	0.097 a	42.0 a
	4	0.585 a	3.0 b	0.105 a	36.0 b
	5	0.607 a	3.0 b	0.098 a	35.0 b
	6	0.592 a	2.0 c	0.103 a	29.7 c

3.4. Effect of maturity stage and inoculum concentration on the incompatible interaction at 20 °C

P. digitatum was not able to develop rot in 'Golden Smoothee' apples at any harvest date and at any inoculum concentration studied. However, a small number of apples at harvests 4–6 showed infection at 10^7 conidia/mL inoculum concentration but the decay was limited to the initial infection site (Fig. 3D–F).

A prominent reaction was observed in the peel and in the pulp (dead) of the fruits when *P. digitatum* did not infect apples. That reaction was most prominent in immature apples at 10^7 conidia/mL inoculum concentration (Fig. 3A–C) and showed a concentration-dependent profile.

Both immature and commercially mature fruits inoculated with *P. digitatum* showed an additional reaction in the peel: a yellow (degreened) circle around the infection site (Fig. 3A and D). That reaction appeared irrespective of whether *P. digitatum* was able to infect the fruit or not.

3.5. Effect of maturity stage and inoculum concentration on the incompatible interaction at 0 °C

At 0 °C storage temperature, *P. digitatum* was not able to develop infection regardless of inoculum concentration or maturity stage of fruit. The reaction behaviour was the same than that obtained at 20 °C but the reaction was less intense (data not shown).

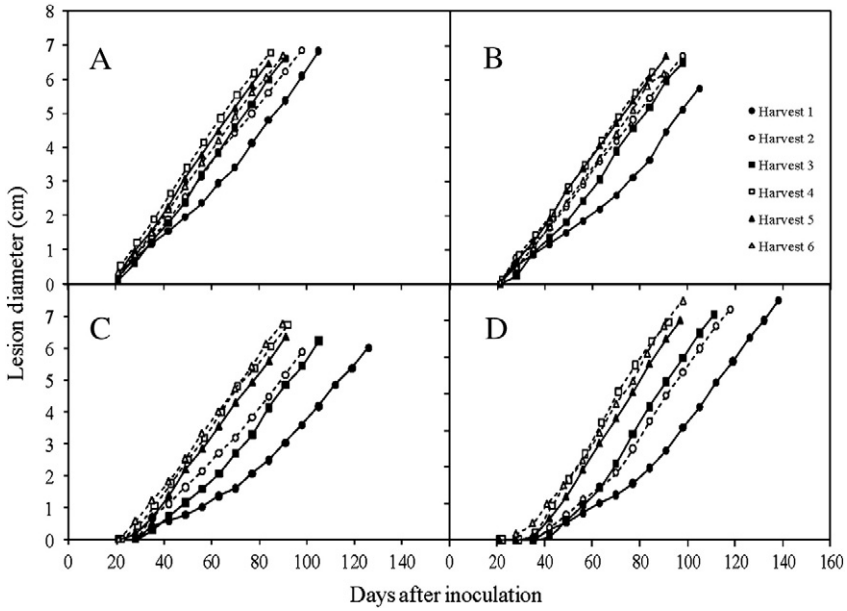

Fig. 2. Influence of maturity stage on lesion diameter (cm) in the compatible interaction at 0 °C and 85% HR storage conditions. "Golden Smoothee" apples were harvested at six different dates and inoculated with *Penicillium expansum* at four different inoculum concentrations: 10^7 conidia/mL (A), 10^6 conidia/mL (B), 10^5 conidia/mL (C) and 10^4 conidia/mL (D). Each point represents the mean of 20 fruit.

3.6. Histochemical results

The Maüle test resulted in a typical orange-reddish-brown staining in the epicarp cells. In this study, the samples with a positive Maüle reaction showed only a brown staining and a positive reaction was found around the wound in control samples over either short- (data not shown) or long-period response (Fig. 4A–C) but only in immature apples. For apples inoculated with *P. expansum* at 10^4 and 10^7 conidia/mL, the Maüle reaction was positive only for the immature harvest at the short-period response (1 day after inoculation) (data not shown).

At immature harvest and at the short-period response, the Maüle reaction was positive for *P. digitatum* inoculated at 10^7 and 10^4 conidia/mL, and the reaction intensity was correlated with the pathogen concentration (data not shown). In general, intensity of the Maüle reaction was lower in the short-period response to *P. digitatum* as maturity advanced. In contrast to *P. expansum* samples (which rotted), wounds inoculated with *P. digitatum* could be analysed at 7 days after inoculation. The Maüle test showed an important reaction around inoculated wounds in immature and in mature fruits (Fig. 4G and H) but over-mature fruits did not show reaction (Fig. 4I). Samples infected with *P. digitatum* at 10^4 conidia/mL (Fig. 4D–F) showed lower intensity than at 10^7 conidia/mL (Fig. 4G–I).

Lignified cells could be also identified with the Toluidine blue O test as violet-stained cells. A positive reaction for lignin was also obtained with the toluidine blue O test and the results were similar to the Maüle reaction when it appeared but at short-period of response the reaction was easier to detect (Fig. 5). Moreover, this stain reveals a brown colour when rot starts (Fig. 5E) but the visual signs of rot development were not evident.

Sudan IV and aniline blue reagents for the detection of suberin and callose, respectively, did not show positive reactions in these histochemical assays for any samples studied (control, *P. expansum* and *P. digitatum*; data not shown).

4. Discussion

The infection capacity of *P. expansum* and *P. digitatum* in apples at different maturity stages, different inoculum concentrations and two

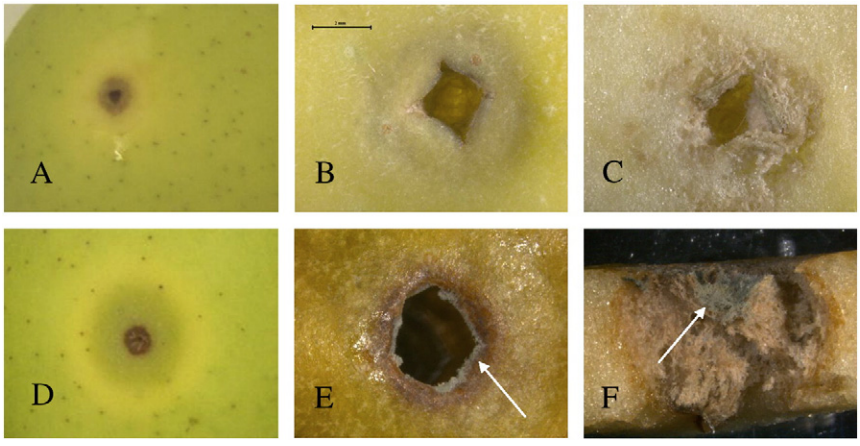

Fig. 3. "Golden Smoothee" apples inoculated with *Penicillium digitatum*. A visible reaction around the inoculation site was found in immature apples inoculated at 10^7 conidia/mL inoculum concentration (A–C). *P. digitatum* at 10^7 conidia/mL inoculum concentration was able to infect a small group of apples from commercial harvests (D–F) but was not able to develop decay. The reaction in the peel (B and E) and in the pulp (C and F) is shown with a stereoscope magnification of 12.5×. Scale bar = 2 mm. Arrows pointed out rot at the infection site.

storage temperatures were studied in this work. *P. expansum* (compatible pathogen) showed infection at all conditions assayed, whereas *P. digitatum* (incompatible pathogen) was only able to infect some fruits under very specific conditions (commercial and over-mature fruit) and at the highest inoculum concentration used. However, the decay was limited to the initial infection site.

P. expansum is a pathogen with a very broad host range and can cause diseases in 21 genera of plants (Li et al., 2010). In the present study we show a broad capacity to infect apples under a wide range of favourable and unfavourable conditions. Fruit maturity appears to be important in determining the resistance of apples to *P. expansum* because over-mature fruit are more susceptible to infection than immature fruit (Neri et al., 2010; Torres et al., 2003).

Our results using *P. expansum* (compatible interaction) indicated that immature harvests exhibit a slower rate of lesion development in comparison to the other harvests both at 20 and 0 °C storage temperatures. Moreover, the differences were greater when lower inoculum concentrations were used. At 20 °C, Su et al. (2011) demonstrated that susceptibility of apples to *Botrytis cinerea* increased with fruit maturity. However, the significant differences that they obtained were between lesion diameters in the late harvest compared with optimal and early harvests but non significant differences in the percentage of decay were obtained among harvests. These results agree with ours because maturity affects growth rate development but not lesion incidence. In another compatible pathosystem (oranges-*P. digitatum*), Vilanova et al. (2012) found that *P. digitatum* growth rate in immature harvests had a slow development in relation to over-mature harvests; however, when the fruit was stored at cold temperature, no significant differences at the highest inoculum concentration assayed were found. On the other hand, Boonyakiat et al. (1987) and Spotts (1985) showed that in pears inoculated with *P. expansum* and stored at cold temperature, the incidence in immature and mature fruits was lower than in over-mature fruits.

Growth rate and visible symptoms of decay both appeared influenced by cold temperatures. Shortened lag phases and increased growth rates were found when the temperature increased to the optimum. Our results are in agreement with that obtained by Baert et al. (2007a,b) in apples inoculated with *P. expansum*. Moreover, in *in vitro* assays, Gougouli and Koutsoumanis (2010) found that the lowest storage temperature at which *P. expansum* growth was observed was −1.3 °C and at this temperature a very slow increase of the mycelium diameter after an extensive lag period of about 1 month was observed. Similar results were obtained by Plaza et al. (2003) with other *Penicillium* species such as *P. digitatum* and *P. italicum*.

At 0 °C no significant differences were found in growth rate between inoculum concentrations at any harvest date; however, the greenest harvest showed significant differences between inoculum concentrations at 20 °C. Similar results obtained Morales et al. (2008) in *P. expansum*-apples who found significant differences only in growth rates between inoculum concentrations at 20 °C. However, Baert et al. (2008) in the same pathosystem and García et al. (2010) in *P. expansum in vitro* assays showed that inoculum levels did not affect significantly the rate of growth whereas lag phases increased independently of the temperature. These differences could be due to the fact that wound-healing process at 20 °C is more active than at 0 °C and the greenest harvest inoculated with the lowest inoculum concentration are the most adverse conditions tested.

To assess the maturity of the fruit it is usual to measure different quality parameters. In our study we found significant differences for all of them between harvests; however, any of the studied parameters could be directly correlated with rot susceptibility.

Despite the fact that *P. digitatum* is a very specific pathogen that only infects citrus fruit, in this work surprisingly, we found that can infect apples but the decay was limited at the initial infection site. To our knowledge, this is the first work that reports the capacity of this non-host pathogen to infect apples. Macarisin et al. (2007) showed the capacity of *P. expansum* (non-host pathogen) to germinate and temporarily grow in citrus fruit. However, more recent studies from Vilanova et al. (2012) showed that *P. expansum* could develop rot in oranges under determinate maturity stages, inoculum concentration and temperature.

At 0 °C no signs of infection of *P. digitatum* were found at any of the studied conditions. These results could be explained because *P. digitatum* can germinate and grow in the range 4–30 °C and the

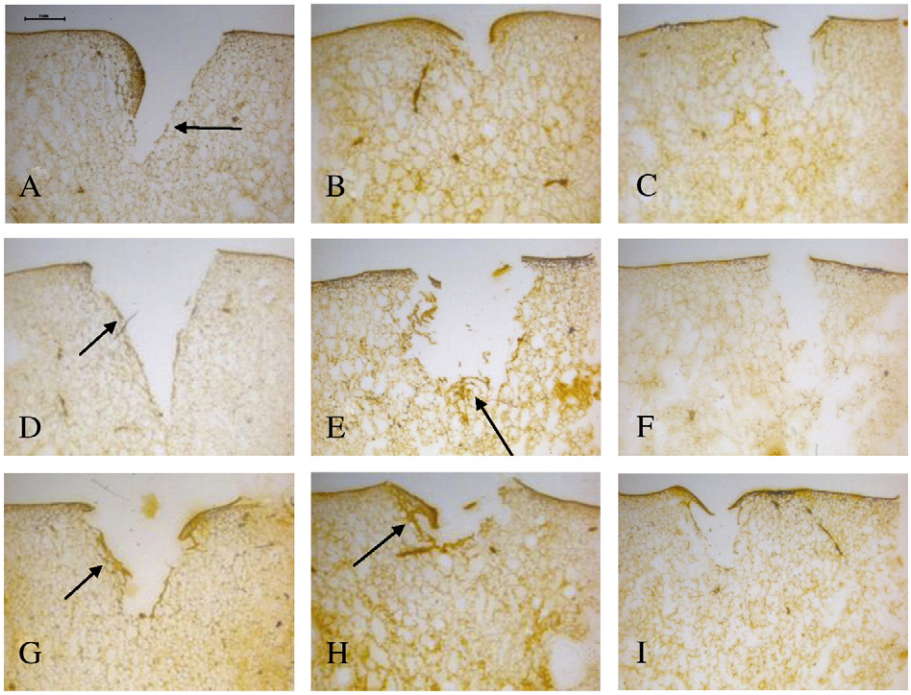

Fig. 4. Maüle tests for lignin in control (A–C), *Penicillium digitatum* at the 10^4 conidia/mL concentration (D–F) and *P. digitatum* at the 10^7 conidia/mL concentration (G–I) 7 days after inoculation and in immature (A, D and G), mature (B, E and H) and over-matured (C, F and I) oranges. Maüle tests resulted in typical orange-reddish-brown stains around the wounds, which is a positive sign for the presence of lignin compounds. Stereoscope magnification: 20×. Scale bar = 1 mm. Arrows pointed out lignin deposits.

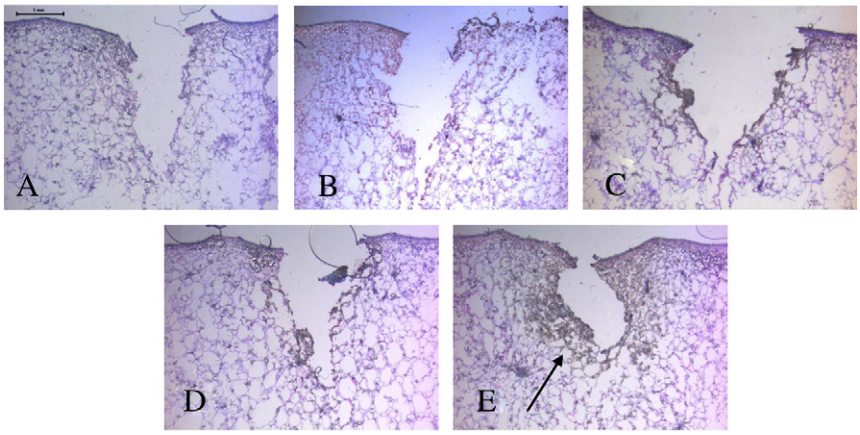

Fig. 5. Toluidine tests for lignin at the short-period response in control (A), *Penicillium digitatum* at the 10^4 conidia/mL concentration (B), *P. digitatum* at the 10^7 conidia/mL concentration (C), *Penicillium expansum* at the 10^4 conidia/mL concentration (D), *P. expansum* at the 10^7 conidia/mL concentration (E) 24 h after inoculation and in immature apples. Toluidine blue O tests resulted in typical violet stains around the wounds, which is a positive sign for the presence of lignin compounds. Stereoscope magnification: 20×. Scale bar = 1 mm. Arrow pointed out lignin deposits.

germination delayed and slowed down when the temperature decreased (Plaza et al., 2003). Different behaviour was obtained by Vilanova et al. (2012) in the incompatible interaction *P. expansum*-oranges that showed higher decay incidence and severity at 4 °C than at 20 °C. These results could be explained because *P. expansum* is perfectly adapted to cold temperatures and at 4 °C fruit wound-healing processes and defence mechanisms are slower than at room temperature. On the contrary, *P. digitatum* is not well adapted to cold temperatures.

In the peel and in the pulp a host response (HR) was observed when the non-host pathogen cannot infect the apples. HR is commonly used as a visual marker for incompatible plant-pathogen interactions (Mysore and Ryu, 2004) and it is recognised as brownish and necrotic areas in the host tissue. In studies in leaves of coffee (Silva et al., 2002) and in melon (Romero et al., 2008), a rapid cell death was observed in the incompatible interaction. Similar results were also obtained in orange fruit (Macarisin et al., 2007; Vilanova et al., 2012). However, in our results and in the results above mentioned in oranges, the non-host pathogen could infect the fruit under determined conditions. These results could be attributed to the hypersensitive reaction against necrotrophic fungi that in some cases do not prevent the pathogen growth development or even could stimulate it (Govrin and Levine, 2000).

The HR is the result of very complex events and is related with the cell wall reinforcement (by the deposition of physical barriers) (Mysore and Ryu, 2004) and to identify these possible compounds, histochemical studies were performed. Both tests used to detect lignin production showed positive reaction at short-period response (1 day) in both compatible and incompatible interactions. These results are similar than the obtained by Vilanova et al. (2012) in oranges and could indicated that lignin-like substances appeared for the action of a pathogen infection irrespective of whether the pathogen is compatible or incompatible. Lignification was analysed in oranges inoculated with the non-host pathogen in the long-period response (7 days), and the reaction obtained at this time was more intense than at 1 day. Moreover, a more important reaction was obtained in immature and commercial fruits than in over-mature fruits. These results agreed with those obtained by Su et al. (2011) in apples, who showed that the lignin quantity measured gravimetrically was higher in wounded tissue from early harvested fruit in comparison with that from late harvest fruit.

The other histochemical tests used for suberin and callose did not show a positive reaction at any sample analysed as was previously observed in oranges (Vilanova et al., 2012).

This study demonstrates that the infection capacity in compatible and incompatible pathogen–host interaction could be affected by the maturity stage of fruit. Moreover, we found that lignin substances synthesized and cell death in the hypersensitive response could function as defence mechanisms against both *P. expansum* and *P. digitatum*. However, that reaction cannot prevent *P. expansum* development. Until now, no studies about the incompatible interaction in apples with *P. digitatum* have been performed and from our knowledge this is the first time that *P. digitatum* has been shown to infect apples under determined conditions.

Acknowledgements

The authors are grateful to the Spanish Government for financial support by a national project AGL2008-04828-C03-01/AGR (Plan Nacional de I+D+I, Ministerio de Ciencia e Innovación, Spain), for a Ramon y Cajal contract (Ministerio de Ciencia e Innovación, Spain) (Dr. R. Torres) and the 'Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria' (INIA) for L. Vilanova PhD grant. We wish to thank Dr. Ana Maria Jauset for allowing work in her laboratory.

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